

Methodological advances in auxin and cytokinin biology.

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Introduction

The concept of plant hormones as chemical messengers that control plant growth and development is not a new one. Already in 1758, Duhamel du Monceau's experiments suggested communication between plant organs and showed that sap moving from the leaves controls root growth (du Monceau, 1758). More than a century later Julius von Sachs proposed that plants produce “organ-forming substances” - molecules moving to different parts of the plant where they control initiation and development of specific plant organs (von Sachs, 1880). Finally, Charles and Francis Darwin, with their experiments on phototropism of coleoptiles (described in "The Power of Movement in Plants" (Darwin, 1880)) that later led to the discovery of auxin by Went (1928), fully launched the modern research in plant growth substances.

The first note about cytokinin comes from 1913 when Gottlieb Haberlandt observed that compounds from phloem could stimulate cell division in potato parenchyma cells (Haberlandt, 1913). In the 1950s, kinetin, an active compound stimulating cell division, was isolated from herring sperm (Miller et al., 1956). The first naturally occurring cytokinin in plants named zeatin was isolated from immature maize endosperm (Letham, 1973).

Since these initial discoveries, a great number of studies have demonstrated an essential role of both auxin and cytokinin in the regulation of many aspects of plant growth and development including embryogenesis (Friml et al., 2003; Müller and Sheen, 2008), postembryonic organogenic processes such as root (Fukaki et al., 2002; Benková et al., 2003; De Smet et al., 2007; Laplace et al., 2007; Bielach et al., 2012), and shoot branching (Leyser, 2009; Shimizu-Sato et al., 2009; Müller et al., 2015), root (Friml et al., 2002; Blilou et al., 2005; Dello Ioio et al., 2008; Růžicka et al., 2009) and shoot apical meristem activity and phyllotaxis (Reinhardt et al., 2003; Zhao et al., 2010; Yoshida et al., 2011; Chickarmane et al., 2012) vasculature development (Mähönen et al., 2006a; Hejácíko et al., 2009; Bishopp et al., 2011b) as

well as tropic responses (Rouse et al., 1998; Müller et al., 1998; Luschning et al., 1998). Importantly, a classic series of experiments by Skoog and Miller (1957) demonstrated that the ratio of cytokinin to auxin profoundly influences the morphogenesis of roots and shoots in plant tissue culture. This was one of the first studies revealing auxin and cytokinin interaction in the differentiation of plant organs and pointed at hormonal cross-talk as an important aspect of auxin and cytokinin regulatory functions (reviewed in Moubayidin et al., 2009; Depuydt and Hardtke 2011; Schaller et al., 2015).

Nevertheless, it has been primarily the recent boom of modern technologies and approaches including analytical chemistry, biochemistry, molecular biology, genetics, cell and developmental biology that have enabled rapid progress in deciphering the auxin and cytokinin activities at the molecular level. Due to ongoing improvements and development of new methods, we are gaining deeper insights into mechanisms that control auxin and cytokinin biosynthesis, distribution, perception and signal transduction as well as insights into their functions in the regulation of plant growth and development. In this review, we shall briefly discuss the major recent progress made in this area, and highlight the importance of continuous methodological improvements.

1. Discovery of auxin and cytokinin

Discovery of auxin is tightly linked with Darwin's early studies on coleoptiles. Based on the bending of coleoptiles toward unilateral light, the existence of a messenger molecule named auxin (from the Greek "auxein" meaning "to grow") was predicted, which was apparently transported from the site of light perception at the tip of coleoptile towards the site of response where bending occurs (Darwin 1880). Later, it was demonstrated that an asymmetric accumulation of auxin at the non-illuminated side compared to the illuminated side correlated with differential cell growth and organ bending (Boysen-Jensen, 1911). A model implementing a role for auxin and its asymmetric distribution in the regulation of plant tropic responses was proposed (Cholodny, 1927, 1928; Went, 1928). Although the existence of auxin as a molecule controlling plant growth had been predicted already by Darwin in 1880, its chemical identity remained unknown for a long time. In 1928 Went succeeded in capturing this growth substance from coleoptile tips into agar blocks and demonstrated its biological activity (Went, 1928).

However, due to insufficient analytical methods for detecting low amounts of the hormone, the first auxin (indole-3-acetic acid, IAA) was purified from human urine and culture filtrates of several fungi, both of which are rich sources of substances with auxin activity when tested in the bioassays (Kögl et al. 1934; Thimann and Koepfli 1935). A decade later IAA was eventually discovered in a plant (*Zea mays*) (Haagen-Smit et al. 1946).

The first experimental indication of the existence of cytokinins was reported by Gottlieb Haberlandt (1913), who observed that phloem sap can stimulate division of potato parenchyma cells. Further studies showed that compounds which trigger cell division are present in various other plant species (van Overbeek, 1941; Jablonski and Skoog, 1954). The first molecule with the ability to promote cell division was purified from autoclaved herring sperm DNA. The compound 6-(furfurylamino) purine was named kinetin, and although it is one of the most biologically active cytokinins, it is formed as a DNA degradation product and is not detected in plant tissues (Miller et al., 1955; Hall and de Ropp, 1955). The first naturally occurring cytokinin, zeatin, was almost simultaneously isolated from *Zea mays* by Miller (1961) and Letham (1963). Since then, many naturally occurring cytokinins have been isolated and found to be ubiquitous to all plant species (Salisbury and Ross, 1992).

The discovery and identification of auxin and cytokinins triggered the interest of researchers, who then diversified to explore pathways that underlie auxin and cytokinin biosynthesis and metabolism, their distribution, as well as perception and signal transduction of these two plant hormones. The establishment of *Arabidopsis thaliana* as a model organism for plant molecular biology was one of the important milestones in hormone molecular biology. The use of *Arabidopsis* for mutant screens based on sensitivities to auxin and cytokinin enabled the identification of genes and pathways controlling their metabolism, transport, perception and signaling. These in combination with novel technologies and approaches, such as large scale transcriptome profiling, proteomics, chemical genomics, and most recently mathematical modelling, resulted in major breakthroughs in our understanding of auxin and cytokinin biology.

2. Auxin and cytokinin: insights into biosynthesis.

Although IAA had been recognized as the main native auxin already in 1935 (Thimann, and Koepfli), the question as to how auxin is synthesized remained unanswered for more than 70 years afterwards. Using genetic and biochemical tools, it has been found that IAA is mainly

synthesized from L-tryptophan (Trp) via indole-3-pyruvate (IPA) in a two-step reaction catalysed by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and YUCCA (YUC) (Figure 1a). The TAA family of amino transferases which mediate the first step of the pathway was isolated from independent genetic screens for mutants affected in shade, ethylene, and responses to the auxin transport inhibitor NPA (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009). Severe auxin deficient phenotypes (in developmental processes such as embryogenesis, seedling growth, flower development, vascular patterning, root branching, tropisms, and shade avoidance) as well as reduced endogenous auxin levels were observed in mutants lacking activity of TAA1 and the homologous TAR1 and TAR2, which indicated their function in auxin homeostasis maintenance (Stepanova et al., 2008). The phenotypic defects observed in TAA1/TAR deficient mutants were partially rescued by auxin, whereas induction of TAA1 led to the accumulation of endogenous IPA. Importantly, the recombinant TAA1 protein has been found to catalyse the conversion of Trp into IPA *in vitro* thus providing evidence for its direct involvement in auxin biosynthesis (Stepanova et al., 2008; Tao et al., 2008).

Similarly to *TAA1*, *YUC* genes were originally identified by a genetic screen in *Arabidopsis*. Using an activation-tagged mutant library, a flavin-containing monooxygenase *YUC1* was isolated. The *YUC1* (*yuc1D*) gain-of-function mutant exhibits increase in endogenous IAA and phenotypic alterations mimicking high auxin activity. Disruption of several *YUC* genes in *Arabidopsis* leads to defects in embryogenesis, seedling growth, flower development, and vascular pattern formation (Cheng et al., 2006, 2007). The developmental defects of the loss-of-function *yuc* mutants are rescued by the bacterial auxin biosynthesis gene *iaaM*, supporting *YUC* genes function in auxin biosynthesis (Cheng et al., 2006).

Although previously proposed to act in two independent pathways, recent genetic and biochemical studies showed that the TAAs and YUCs catalyse two consecutive reactions in the same pathway that converts Trp to IAA. Multiple lines of evidence support this model including similarities of both *taa* and *yuc* mutants phenotypes (Won et al., 2011) and enhancement of the auxin related phenotypes when both *YUC* and *TAA* are overexpressed in the same plants (Mashiguchi et al., 2011). Additionally, the *YUC* auxin overproduction phenotypes are suppressed in the *taa* mutant backgrounds, indicating that TAA acts upstream of YUC-mediated auxin biosynthesis (Won et al., 2011). Direct measurement of IPA levels reveals that *yuc* mutants accumulate IPA whereas *taa* mutants are partially IPA deficient, suggesting that TAAs

catalyses synthesis of IPA which is converted by YUCs to IAA (Mashiguchi et al., 2011; Won et al., 2011). Finally, *in vitro* biochemical assays have demonstrated that TAA can convert Trp to IPA and that YUCs produce IAA using IPA as a substrate (Mashiguchi et al., 2011).

Early physiological studies on auxin biosynthesis suggested that auxin is primarily synthesized in the young developing organs such as leaves, shoot apical meristems, and developing fruits and seeds (Bartel, 1997; Ljung et al., 2001). The expression pattern of *TAA* and *YUC* genes modifies this established view on auxin biosynthesis. Local auxin production seems to take place in very distinct cell types, including root and apical embryo meristems, the root cap, quiescent centre (QC), root proximal meristem, vasculature of hypocotyls, as well as apical hooks, thus hinting at the spatio-temporal control of the IAA biosynthesis throughout plant growth and development (Chent et al., 2006, 2007; Stepanova et al., 2008; Tao et al., 2008). Several transcription factors which control *TAA* and *YUC* genes expression have been identified and thus might determine spatio-temporal pattern of the IAA biosynthesis. *LEAFY COTYLEDON2 (LEC2)* (Stone et al., 2008) *SHORT INTERNODES/ STYLISH (SHI/STY)* (Eklund et al., 2010), *PHYTOCHROME-INTERACTING FACTORs (PIFs)* (Franklin et al., 2011; Sun et al., 2012), *INDETERMINATE DOMAIN (IDD)* (Cui et al., 2012) and *PLETHORA* family members (Pinon et al., 2013) have been reported as transcriptional activators of *YUC* and *TAA1* genes. In contrast, the *SPOROCTELESS/NOZZLE (SPL/NZZ)* transcription factor, has been shown to negatively regulate some of *YUC* genes (Li et al., 2008).

Chemical biology-based studies provided additional support for the central role of the IPA pathway in IAA production. Chemical screens for auxin inhibitors uncovered L-kynurenine and L-amino-oxyphenylpropionic acid (L-AOPP) as TAA inhibitors and yucasin as a YUC inhibitor. Application of these compounds reduces endogenous IAA levels and results in phenotype alterations mimicking mutants deficient in auxin biosynthesis (Soeno et al., 2010; He et al., 2011; Nishimura et al., 2014).

Overall, genetic and biochemical analyses support the YUCs/TAAs mediated auxin biosynthesis as the major pathway used to produce auxin during plant development, whereas other pathways catalysed by CYP79B2/B3, nitrilases, aldehyde oxidases, and pyruvate decarboxylases might not be the main pathways in auxin biosynthesis (Zhao, 2012).

The great progress in elucidation of the cytokinin biosynthesis pathway occurred almost 20 years after identification of the chemical nature of cytokinins by Miller (1961) and Letham

(1963). In 1978 Taya and co-workers reported biosynthesis of free cytokinins *in vitro* and demonstrated that cell-free extracts of the slime mold *Dictyostelium discoideum* converts adenosine monophosphate (AMP) and dimethylallyl pyrophosphate (DMAPP) to the active cytokinin iPMP (N6-(D2-isopentenyl)adenosine-5'-monophosphate (Taya et al., 1978). Subsequently, the *ISOPENTENYLTRANSFERASE* (*IPT*) gene from *Agrobacterium tumefaciens* was shown to encode an enzyme with similar activity (Akiyoshi et al., 1984). Later, nine *IPT*-homologues genes were identified by an *in silico* search in the *A.thaliana* genome. The expression of *IPT* genes (except *AtIPT2* and *AtIPT9*) in *E. coli* resulted in the secretion of the cytokinins isopentenyladenine (iP) and zeatin, confirming their function as cytokinin biosynthetic enzymes (Takei et al., 2001). *IPT* genes display distinct, tissue-specific patterns of expression, indicative of cytokinin production sites (Miyawaki et al., 2004; Takei et al., 2004a). Free iP-riboside generated via the *IPT* pathway, as well as the corresponding base, are further stereospecifically hydroxylated to trans-zeatin forms. The *CYP735A1* and *CYP735A2* encoding cytochrome P450 monooxygenases with cytokinin trans-hydroxylase enzymatic activity were identified in *A.thaliana* by a screen employing an (*AtIPT4*)/P450 co-expression system in *Saccharomyces cerevisiae* (Takei et al., 2004b).

The final step in cytokinin biosynthesis, conversion of the cytokinin ribotides to their active, free base forms is catalyzed by the cytokinin nucleoside 5'-monophosphate phosphoribohydrolase LONELY GUY (LOG). These were first identified in rice by a genetic screen for defects in the maintenance of shoot meristems (Kurakawa et al., 2007). In *A.thaliana*, seven homologous genes that encode active LOG enzymes were detected. The *LOG* genes are differentially expressed in various tissues during plant development. (Kuroha et al., 2009). In accordance with their predicted function the conditional overexpression of *LOGs* in *Arabidopsis* reduced the content of iP riboside 5'-phosphates and increased the levels of iP and the glucosides (Kuroha et al., 2009). Alternatively, the cytokinin ribotides are dephosphorylated to the ribosides and subsequently converted to free-base cytokinins (Chen and Kristopeit 1981a, 1981b), however the corresponding genes have not yet been identified (Figure 1b and c).

Levels of active cytokinins in plant cells are tightly controlled. They might be either converted to storage forms through conjugation to glucose (Martine et al., 1998; Hou et al., 2004) or inactivated through irreversible cleavage by cytokinin oxidases (Werner et al., 2001; Werner et al., 2003), (Figure 1c). Development of highly sensitive analytical methods were instrumental in

the detection of numerous cytokinins metabolites and in deciphering complex cytokinin metabolism, followed by identification of the corresponding metabolic enzymes and genes (Letham and Palni 1983; Mok and Mok 2001; Tarkowski et al., 2009).

3. Transport of auxin and cytokinin

By definition, hormones are chemical messengers that are transported to distant tissues and organs to regulate their physiology and development. Darwin's early experiments on coleoptiles had already indicated that controlled transport of auxin from the tip of coleoptile to the bending region might be an essential part of the mechanism through which auxin executes its regulatory function. Later, based on the transport studies, it was proposed that cytokinins and auxin are synthesized only in root tips and shoot apices, respectively, and translocated to target tissues. Although the recent detailed investigations of expression patterns of auxin and cytokinin biosynthesis genes questions this over-simplified model, the tight control of hormone distribution through organs and tissues is considered to be the crucial component of their regulatory mechanisms. Nowadays, the broadly accepted concept is that both hormones are synthesized and act at various sites in a plant body and that they have coordinated functions as long-distance messengers as well as local signals.

The classical transport assays using radioactively labeled auxins outlined main routes of auxin movement in plants (Morris and Thomas, 1978). To transport auxin, plants use two distinct pathways: a non-polar passive distribution through phloem and an active cell-to-cell polar auxin transport (PAT). In the first pathway, most of the auxin and auxin derivatives are rapidly transported via unregulated flow in the mature phloem over long distances in both basipetal and acropetal directions (Nowacki and Bandurski, 1980). The second pathway is slower and acts over shorter distances, transporting auxin in a cell-to-cell manner from the shoot towards the root. In contrast to phloem transport, PAT is specific for active free auxins, occurs in a cell-to-cell manner and is strictly unidirectional. The main PAT stream from the apex towards the root occurs in the cambium and the adjacent partially differentiated xylem elements (Morris and Thomas, 1978; Lomax et al., 1995). In roots, the auxin stream continues acropetally towards the root tip, where part of the auxin is redirected backwards and transported through the root epidermis to the elongation zone (Rashotte et al., 2000).

Based on the chemical nature of auxin and the physiology of PAT, the model of cell-to-cell auxin transport has been proposed, known as the chemiosmotic hypothesis (Rubery and Sheldrake, 1974; Raven, 1975). As a weak acid, a fraction of IAA exists in the acidic environment of the apoplast as the protonated, neutral form (IAAH), which may diffuse through the plasma membrane. In the more basic cytosol, auxin becomes deprotonated (IAA⁻) and is unable to pass passively through the plasma membrane. The chemiosmotic hypothesis predicted that the exit of auxin anions from the cell is mediated by active efflux carriers and that the passive diffusion of auxin can be further facilitated by influx carriers. The polar membrane localization of the auxin efflux carriers in a file of adjacent cells would determine directionality of the auxin flow (Figure 2a).

It has been primarily genetic studies that led to discovery of genes required for auxin influx and efflux (Bennett et al., 1996; Gälweiler et al., 1998; Luschnig et al., 1998; Geisler et al., 2005; Cho et al., 2007). An auxin influx transporter *AUXIN RESISTANT1* (*AUX1*), encoding an amino acid permease-like protein, was found in a screen for auxin resistant plants (Pickett et al., 1990). Strong insensitivity to membrane-impermeable auxin (2,4-D) suggested that the *aux1* mutation interferes with auxin uptake (Bennett et al., 1996), which was confirmed by the transport assays using a *Xenopus* oocyte expression system (Yang et al., 2006). The *A.thaliana* genome encodes four auxin influx transporters: *AUXIN RESISTANT1* (*AUX1*) and three *Like AUX1* (*LAX1*, *LAX2*, *LAX3*) (Parry et al., 2001; Swarup et al., 2008; Péret et al., 2012). Thorough exploration of mutants lacking *AUX1/LAX* activity revealed the essential role of the auxin uptake in the regulation of gravitropism, phototropism, root branching, phyllotaxis, and root hair development (Bennett et al., 1996; Bainbridge et al., 2008; Stone et al., 2008; Swarup et al., 2008; Jones et al., 2009; Péret et al., 2012).

Genetic screens were also instrumental in identifying molecular components of auxin efflux. In the early nineties, the *A.thaliana* mutant, *pin-formed1* (*pin1*) with needle-like inflorescence was described. The characteristic phenotype similar to wild type plants treated with chemical inhibitors of auxin efflux indicated defects in auxin transport. Auxin transport assays in *pin1* stem segments confirmed severe reduction of the basipetal flow of auxin and pointed to a function for PIN1 in auxin efflux (Okada et al., 1991). Indeed, identification of the mutant locus revealed that *PIN1* encodes a putative transmembrane protein with a predicted topology of transporter proteins (Gälweiler et al., 1998). Auxin transport assays in *Arabidopsis* and tobacco

cell suspension culture as well as in heterologous non-plant systems including yeast, mammalian HeLa cells and *Xenopus* oocytes have provided evidence for an auxin efflux capacity of PIN proteins (Petrášek et al., 2006; Yang and Murphy, 2009; Barbez et al., 2013; Zourelidou et al., 2014). The *Arabidopsis* PIN gene family consists of eight members (Zažímalová et al., 2007; Adamovski and Friml 2015). Based on the localization and domain organization, these were divided into two groups. The first group consists of PIN1, PIN2, PIN3, PIN4 and PIN7 and are located at the plasma membrane. The second group comprising PIN5, PIN6, and PIN8 have a reduced middle hydrophilic loop and are located at the endoplasmic reticulum (ER), where they presumably control auxin flow between the cytosol and ER lumen, thus possibly affecting subcellular auxin homeostasis (Mravec et al., 2009; Ding et al., 2012). Similarly, PIN-LIKES proteins (PILS) are located in the ER and might play a role in regulation of intracellular auxin homeostasis (Barbez et al., 2012) (Figure 2).

In addition to the PIN family of plant-specific auxin transporters, plant orthologs of the mammalian ATP-binding cassette subfamily B (ABCB)-type transporters of the multidrug resistance/phosphoglycoprotein (ABCB/MDR/PGP) protein family (Noh et al., 2001; Verrier et al., 2008) have been implicated in auxin transport. Biochemical evidence for the ABCB proteins auxin transport activity has been demonstrated both in plant and non-plant systems. In contrast to polar localization of PINs, which corresponds with known direction of auxin flow, the ABCBs presumably act in nondirectional long-distance auxin transport controlling amount of auxin in these streams (Noh et al., 2001; Verrier et al., 2008; Peer et al., 2011).

The chemiosmotic hypothesis predicted that the polar membrane localization of auxin transporters determines the directionality of the auxin flow. This concept was supported by observations of a polar subcellular localization for PIN proteins (Gälweiler et al., 1998; Luschnig et al., 1998) and a tight correlation between PIN polarity and directions of auxin flow (Wisniewska et al., 2006). Phosphorylation of PINs controlled by a set of kinases and phosphatases (Benjamins et al., 2001; Friml et al., 2004; Michniewicz et al., 2007; Zhang et al., 2010; Huang et al., 2010; Zourelidou et al., 2014), Ca^{2+} signaling (Zhang et al., 2011), cell wall (Feraru et al., 2011) or mechanical signals orienting the plant microtubule network (Heisler et al., 2010) were found to determine PIN protein activity and polarity. Cell-biological studies revealed that PIN auxin efflux transporters may not solely reside at the plasma membrane since they undergo constitutive cycles of endocytosis and recycling back to the plasma membrane (Geldner

et al., 2001; Dhonukshe et al., 2007) (Figure 2c). The constitutive endocytosis and recycling of PIN proteins depends on complex subcellular trafficking machinery including the coat protein clathrin (Dhonukshe et al., 2007; Kitakura et al., 2011; Wang et al., 2013), ADP-ribosylation factor guanine-nucleotide exchange factors ARF - GEFs (Geldner et al., 2001, 2003; Kleine-Vehn et al., 2008a,b; Naramoto et al., 2014); ARF-GTPase-activating protein VASCULAR NETWORK DEFECTIVE3 (Naramoto et al., 2010), the related ARF-GEF GNOM-LIKE1 (Teh and Moore, 2007) and small GTPase Rab1b (Feraru et al., 2012). Downstream of endocytosis, the early endosomal trafficking of PINs is controlled by another ARF-GEF, BFA-visualized endocytic trafficking defective1, and the Sec1/Munc18 family protein BEN2 (Tanaka et al., 2009, 2013). The endocytosis and constitutive recycling of PIN proteins has been implicated in the maintenance of PIN polar localization and as a mechanism for rapid modifications of PIN polarity during various developmental processes including embryogenesis (Friml et al., 2003; Robert et al., 2013), lateral root organogenesis (Benkova et al., 2003; Dubrovsky et al., 2008) or tropic responses (Friml et al., 2002, Kleine Vehn et al., 2010; Ding et al., 2011; Rakusova et al., 2011).

Like auxin, cytokinins are highly mobile molecules. However, in contrast to the well characterized transport machinery of auxin, the nature of cytokinin transport is less clear. Long-distance transport of cytokinin is supported by the discovery of cytokinins in xylem and phloem sap (Gillissen et al., 2000; Burkle et al., 2003; Bishopp et al., 2011a). In xylem sap, the major form of cytokinin is tZ-riboside (tZR) (Beveridge et al. 1997; Takei et al. 2001; Hirose et al. 2008), while in phloem sap iP-type cytokinins, such as iP-ribosides and iP-ribotides are detected (Corbesier et al. 2003; Hirose et al. 2008). Accordingly, grafting experiments between wild-type plants and cytokinin biosynthesis mutants showed preferential transport of different cytokinins; trans-zeatin tZ-type cytokinins were transported from the root to the shoot, while iP-type cytokinins moved from the shoot to the root (Matsumoto-Kitano et al., 2008). Thus, plants might use tZ- type as an acropetal messenger and iP-type cytokinins as basipetal messengers (Kudo et al., 2010). Recently, transport assays using radiolabeled cytokinins confirmed basipetal movement of cytokinin through the phloem and revealed that basipetal transport of cytokinin occurs through symplastic connections in the phloem (Bishopp et al. 2011b). Reverse genetics approaches applied to systematically characterize the ATP-binding cassette transporter proteins in *A. thaliana* yielded the identification of ABCG14 as a transporter involved in the long-

distance acropetal (root to shoot) translocation of the root-synthesized cytokinin. Plasma membrane-located ABCG14 is expressed primarily in the central cylinder of roots and loss of ABCG14 activity interferes with the translocation of tZ-type cytokinins from roots to shoots. *In planta* feeding of radiolabeled tZ suggests that ABCG14 acts as an efflux pump (Zhang et al., 2014).

Mechanisms of cytokinin uptake into cells have been studied using radiolabeled cytokinins in *Arabidopsis* cell cultures. Experiments predicted the presence of proton-coupled high-, medium-, and low affinity cytokinin transport systems (Burkle et al., 2003; Cedzich et al., 2008). So far, the equilibrative nucleoside transporter (ENT) family and the purine permease (PUP) family have been found to facilitate cytokinin transport (Burkle et al. 2003, Li et al., 2003; Hirose et al. 2005). Among *Arabidopsis* PUP family proteins (Gillissen et al., 2000), active uptake of free cytokinin bases and several adenine derivatives by PUP1 and PUP2 was demonstrated using a yeast system (Burkle et al., 2003). Expression of PUP2 in the phloem of *Arabidopsis* leaves suggested a role for PUP2 in phloem loading and unloading for long-distance transport of adenine and possibly cytokinins (Burkle et al., 2003). Among the plant ENT transporters, competitive uptake studies in yeast cells showed that *Arabidopsis* ENT3, ENT6, ENT7 and rice ENT2 can facilitate uptake of iP-riboside and tZ-riboside (Li et al., 2003; Hirose et al., 2005). Furthermore, mutants lacking either ENT3 or ENT8 exhibit reduced cytokinin uptake efficiency (Sun et al., 2005). Distinct expression patterns of *ENT* genes detected in root, leaf, and flower vasculature suggest that they may act differently during plant growth and development (Li et al., 2003; Sun et al., 2005; Hirose et al., 2008), however their function as cytokinin transporters *in planta* needs to be experimentally supported. In summary, in contrast to high substrate specificity of the auxin transport system, translocation of cytokinins *in planta* seems to be mediated through transporters with affinities to a broader spectrum of molecules such as purine derivatives and nucleosides.

4. Perception and signal transduction of auxin and cytokinin.

Solving the puzzle of auxin and cytokinin perception mechanism has been undoubtedly one of the biggest challenges of the last years. Establishment of the *Arabidopsis* genetic model has provided excellent tools to address this long standing question and it has been forward

genetic screens in *Arabidopsis* that have led to the identification of backbone elements of both auxin and cytokinin signal transduction cascades. Genetics in combination with advanced molecular and biochemical approaches enabled the achievement of a comprehensive view on the molecular principles of auxin and cytokinin perception and signal transduction.

Several independent forward genetic screens for mutants insensitive to auxin (Rouse et al., 1998; Ruegger et al., 1997, 1998) and expression profiling to isolate auxin inducible genes (Abel et al., 1995; Hagen and Guilfoyle 2002; Abel and Theologis 1996; Ulmasov et al., 1997) led to identification of all key molecular components required for auxin response such as TIR1 (encoding for F-box component of the E3 ubiquitin ligase SCF^{TIR1/AFBs}), the auxin early inducible *Aux/IAA* genes as well as the ARF transcription factors that recognise auxin response elements in the promoters of the *Aux/IAAs* (Gray et al., 1999; Abel and Theologis 2010). However, how these genes might constitute the pathway sensing and transducing hormonal signal was not obvious. Using advanced genetic and biochemical approaches the auxin signalling circuit has been resolved and TIR1 identified as the auxin receptor. It has been shown that auxin mediates interaction between TIR1/AFBs and *Aux/IAA* proteins which stimulates *Aux/IAAs* ubiquitination by SCF^{TIR1/AFBs} E3-ubiquitin ligases for subsequent degradation by the proteasome. This leads to de-repression of ARFs, and transcriptional regulation of downstream response genes. At low auxin concentration, *Aux/IAAs* form a complex with ARF transcription factors and the transcriptional corepressor TOPLESS (TPL), thus preventing the ARFs from regulating target genes (Gray et al., 2001; Dharmasiri et al., 2005a,b; Kepinski and Leyser 2005; Tan et al., 2007; Szemenyei et al., 2008) (Figure 2b).

Although the framework which outlines the core molecular mechanism of auxin perception and signal transduction has been recognised, the question as to how *TIR1/AFB*, *Aux/IAAs* and *ARF* families, each comprising many homologous members, mediate specific developmental output remains to be answered. As indicated by recent studies, multiple levels of control appear to exist, including spatio-temporal specific expression of individual auxin signalling pathway components (Overvoorde et al., 2005; Okushima et al., 2005), as well as differences in affinities of the TIR1/AFB auxin receptors for the *Aux/IAA* repressors (Calderón-Villalobos et al., 2012; Moss et al., 2015), of *Aux/IAA* repressors for the ARFs transcription factors (Vernoux et al., 2011; Lee et al., 2014; Korasick et al., 2014; Nanao et al., 2014;

Shimizu-Mitao and Kakimoto, 2014), and of ARFs for their binding motifs in promoters of the target genes (Boer et al., 2014), which may allow fine-tuning of auxin responses.

After a period of biochemical attempts in the early 1970s to identify the cytokinin receptors, the forward genetic screens turned out to be successful strategies. In a screen of the activation tagged *Arabidopsis* mutants for cytokinin independent growth, the sensor histidine kinase CKI1 was recovered. This finding suggested that the multi-step phosphorelay similar to bacterial two-component signalling system might underlie the cytokinin signal transduction (Kakimoto, 1996). Another screen for cytokinin insensitive mutants led to identification of the *CRE1* (*CYTOKININ RESISTANT 1*) encoding a sensor histidine kinase related to CKI1 (Inoue et al., 2001). At about the same time, the *WOODEN LEG* (*WOL*) mutant allele of the AHK4/*CRE1* gene (exhibiting severe defects in the vasculature differentiation; Mähönen et al., 2000) was identified, along with the AHK2 and AHK3 homologues required for cytokinin response (Hwang and Sheen et al., 2001; Ueguchi et al 2001; Higuchi et al., 2004; Nishimura et al., 2004). Elegant experiments in yeast and bacteria provided first evidence that *CRE1*/*AHK4* functions as a cytokinin receptor (Inoue et al., 2001; Ueguchi et al., 2001, Suzuki et al., 2001); later corroborated by direct binding assays with radiolabeled cytokinins (Romanov et al., 2005, 2006; Stolz et al., 2011).

Subsequent studies focusing on the downstream signaling cascade revealed that genes with high similarity to molecular elements of the multi-step phosphorelay pathway including sensor histidine kinases (AHKs), histidine phosphotransfer proteins (AHPs) and response regulators (ARRs) are present in the *Arabidopsis* genome (Mizuno, 2005; Schaller et al., 2008). Genetic and biochemical characterization of their functions in the cytokinin response yielded the current model of the cytokinin signalling pathway. In brief, a cascade of auto- and transphosphorylation events triggered by cytokinin leads to activation of AHK receptors and transduction of the signal to downstream components. Downstream of the AHK receptors, the AHPs continuously translocate between cytosol and nucleus to mediate signalling by activating type-B ARABIDOPSIS RESPONSE REGULATORS (ARRs), transcription factors which then trigger the transcription of specific genes. A negative feed-back loop is provided by type-A ARRs, which inhibit the activity of type-B ARRs by an unknown mechanism (Hwang and Sheen, 2001; Sakai et al., 2001; Mason et al., 2005; Hutchison et al., 2006; To et al., 2007, Argyros et al., 2008; Kieber and Schaller, 2014). Furthermore, a family of F-box proteins, called

the KISS ME DEADLY (KMD) family, targets type-B ARR proteins for degradation and attenuates cytokinin pathway activity (Kim et al., 2013) (Figure 3). The large majority of cytokinin receptors localize to the ER, suggesting a central role of this compartment in cytokinin signaling (Caesar et al., 2011; Wulfetange et al., 2011); nevertheless, a small part of the cytokinin receptors might perceive a signal from the plasma membrane (Wulfetange et al., 2011).

Recently, a set of cytokinin-regulated transcription factors named cytokinin response factors (CRFs) have been described as a potential branch emerging from the classical multi-step phosphorelay parallel to that of type-B ARRs (Rashotte et al., 2006). CRFs are members of the AP2/EREBP family of transcription factors, containing a single AP2–DNA binding domain, distinct from both DREB and AP2 proteins. There are eight members of CRF family in *Arabidopsis* (CRF1–CRF8) with CRF7 and CRF8 being atypical as they lack C-terminal extensions (Sakuma et al., 2002; Nakano et al., 2006; Rashotte and Goertzen, 2010). The transcript abundance of certain *CRFs* (*CRF2*, *CRF5* and *CRF6*) is rapidly upregulated by cytokinin (Rashotte et al., 2006). Protein-protein interaction analysis indicated that CRFs are able to interact with each other to form homo- and/or heterodimers as well as with components of the classical cytokinin signaling pathway. Transcriptome analysis has revealed a large overlap in CRFs and type B ARR targets, pointing at a close link between both branches of the cytokinin signaling pathway.

However, how the specificity of cytokinin response is achieved by the signalling cascade, where each step is supported by a gene family comprising several members, awaits further investigation.

Importantly, elucidation of the molecular elements and mechanistic principles of auxin and cytokinin transduction pathways has enabled the development of specific sensors for monitoring auxin and cytokinin *in planta*. Nowadays, highly sensitive reporters such as *DR5* (Ulmasov et al., 1997); DII-VENUS (Band et al., 2012; Brunoud et al., 2012), and *TCS* (Müller and Sheen; 2008) are extensively used for mapping auxin and cytokinin activities, respectively, and demonstrate a great potential of these tools for better understanding of the roles of auxin and cytokinin in plant development.

5. Auxin and cytokinin interaction in regulation of plant development.

Since the initial discovery of auxin and cytokinin, the number of reports supporting their regulatory role in various aspects of plant development has accumulated. Moreover, studies of auxin and cytokinin function in plant cell suspension growth provided the first evidence of hormonal interaction and its role in directing plant development. The experiments of Skoog and Miller (1957) demonstrated that both auxin and cytokinin are not only required to induce and maintain cell division and growth in plant tissue culture, but that the auxin:cytokinin ratio determines distinct organogenic pathways. A high ratio of cytokinin to auxin stimulated formation of shoots, whereas a low ratio induced root regeneration. Tight communication between auxin and cytokinin is crucial for proper establishment of meristems in early embryogenesis (Muller and Sheen, 2008; Su et al., 2011), ovule development (Bencivenga et al., 2012), shoot apical meristem activity and phylotaxis (Reinhardt et al., 2003; Werner et al., 2003; Leibfried et al., 2005; Zhao et al., 2010), shoot and root branching (Domagalska and Leyser, 2011; Laskowski et al., 1995, 2008; Laplaze et al., 2007; Bielach, et al., 2012; Marhavý et al., 2011; 2014), root growth and meristem maintenance (Dello Ioio et al., 2008). Hence the deciphering of molecular and mechanistic bases of auxin and cytokinin interaction became one of the major themes in plant biology. Over the years, research on developmental processes in plants has uncovered genes and networks, giving first insights into molecular mechanisms of auxin and cytokinin cross-talk in the context of these complex developmental programs. Here, a few examples of auxin-cytokinin crosstalk mechanisms and their relevance in coordination of specific developmental processes are discussed.

It has been shown that specification of the root pole during the early phases of embryogenesis is dependent on the tightly balanced activity of auxin and cytokinin. Auxin was found to stimulate expression of the cytokinin signaling repressors *ARR7* and *ARR15* and thus to attenuate the output of the cytokinin pathway. Lack of this auxin-driven negative feedback loop resulted in the up-regulation of the cytokinin response and severe patterning defects at the embryonic root pole (Müller & Sheen, 2008). Interestingly, recent observations hint at another auxin-cytokinin regulatory module acting in the early embryogenesis. Among the transcriptional targets of AUXIN RESPONSE FACTOR (ARF5/MP), previously linked with embryonic root specification (Hardtke and Berleth, 1998; Hamann et al., 2002), TARGET OF MONOPTEROS (TMO3), coding for the CRF2 was identified (Schlereth et al., 2010). Expression of CRF2 and homologous genes is cytokinin responsive and interference with their functions leads to severe

embryonic defects (Rashotte et al., 2006). Furthermore, two auxin efflux transporters (*PIN1* and *PIN7*), both shown to control distribution of auxin during early embryogenesis (Friml et al., 2003), were identified as CRF2 transcriptional targets (Šimášková et al., 2015). However, how these two regulatory circuits jointly coordinate early embryogenesis requires further investigation.

Auxin and cytokinin act in an antagonistic manner to define the root apical meristem size by promoting cell division and differentiation, respectively (Dello Ioio et al., 2007, Růžicka et al., 2009). A complex network of auxin and cytokinin interactions has been implicated in the root meristem activity control. Cytokinin modulates the auxin pathway by affecting the expression of its signaling components. Cytokinin (through the AHK3 receptor and ARR1 and ARR12 response regulators) was shown to directly activate transcription of the auxin repressor *IAA3/SHORT HYPOCOTYL 2 (SHY2)*. This leads to the attenuation of auxin responses and reduced expression of *PIN* auxin efflux transporters (Vieten et al., 2005; Dello Ioio et al., 2008, Pernisová et al., 2009, Růžicka et al., 2009). Consequently, a decreased abundance of PINs limits the auxin supply to the root apical meristem, thereby restricting its meristematic activity (Dello Ioio et al., 2008; Růžicka et al., 2009). Besides this transcription-based regulation of auxin activity and distribution, cytokinin was also found to modulate the endocytic trafficking of PIN1 by redirecting this membrane protein for lytic degradation in the vacuoles. (Zhang et al., 2011; Marhavý et al., 2011). This alternative mode of cytokinin action provides a mechanism for rapid control of auxin fluxes; and as recently suggested, the enhanced depletion of PIN1 at specific polar domains by cytokinin might also modulate direction of the auxin flow (Marhavý et al., 2014).

Another mechanism through which auxin and cytokinin balance each other's activities occurs by a crosstalk between their metabolic pathways. High cytokinin levels promote auxin biosynthesis (Jones et al., 2010) and auxin, in turn, gives feedback on the cytokinin metabolism by inducing CYTOKININ OXIDASE (CKX) thereby decreasing cytokinin levels (Eklöf et al., 1997, Nordström et al., 2004; Carabelli et al., 2007). On the other hand, in the root apical meristem, auxin enhances (in an *IAA3/SHY2* dependent manner) the expression of ISOPENTENYL TRANSFERASE5 (*IPT5*), which encodes a rate limiting enzyme in the cytokinin biosynthesis, eventually resulting in the local up-regulation of cytokinin levels (Dello Ioio et al., 2008, Miyawaki et al., 2004).

Both auxin and cytokinin exhibit specific functions in the shoot apical meristem. High cytokinin promotes proliferation of undifferentiated cells, whereas auxin coordinates organogenesis in the peripheral zone (Schaller et al., 2015). Cytokinin participates in the WUSCHEL/WUS-CLAVATA/CLV, the core regulatory loop controlling shoot apical meristem activity, by stimulating WUS expression (Gordon et al., 2009). By direct repression of the *ARR7* and *ARR15* cytokinin signaling repressors, WUS further reinforces the cytokinin promoting effect on the WUS-mediated pathway (Leibfried et al., 2005). An important additional input in this cytokinin-driven regulation is provided by auxin. In mutants defective in auxin biosynthesis, transport and signaling, expression of *ARR7* and *ARR15* was found to be enhanced, and the *ARF5/MP* transcription factor was identified as a direct repressor of their transcription (Zhao et al., 2010). This constitutes a regulatory circuit in which auxin enhances cytokinin response by attenuating the expression of the cytokinin signaling repressors, and consequently promoting WUS activity in the WUS-CLV loop.

At the peripheral zone of the shoot apical meristem, new organ formation is triggered by auxin (Reinhardt et al., 2003). Studies following pathways regulated by auxin transport and response revealed that initiation of the lateral organs is accompanied by modulations in the polarity of PIN1 and redirection of the auxin towards incipient primordia (Heisler et al., 2005). The accumulation of auxin correlates with a decrease in *SHOOT MERISTEMLESS (STM)* expression, which eventually results in lower cytokinin at the peripheral zone (Hamant et al., 2002). How PIN1 polarization throughout the shoot apical meristem is coordinated and whether cytokinin contributes to the regulation of polar auxin transport through mechanisms analogous to these detected in root is unknown. Nevertheless, a reduced level of PIN1 in the maize *ARR* repressor ortholog mutant *abphyl 1* supports such a scenario (Lee et al., 2009). Recently, Besnard et al. (2014) provide further evidence for cytokinin function in the peripheral zone and coordination of lateral organ initiation. Analysis of *AHP6* expression patterns along with monitoring of auxin and cytokinin sensitive reporters indicates that AHP6, which acts as a repressor of cytokinin signalling (Mähönen et al., 2006), regulates the spatiotemporal pattern of cytokinin activity at the shoot apical meristem periphery. The cytokinin inhibitory fields generated downstream of auxin by AHP6 might stabilize auxin fields, thereby increasing robustness of the phyllotactic patterning (Besnard et al., 2014).

Studies of auxin-cytokinin cross-talk directing other developmental process (including initiation and organogenesis of ovules; vasculature differentiation, shoot and root meristem activity and lateral branching (reviewed in Moubayidin et al., 2009; Depuydt and Hardtke 2011; Schaller et al., 2015) point towards specific as well as common aspects of mechanisms mediating mutual communication between these two hormonal pathways.

With increasing amounts of confirmed molecular interactions and circuits that determine hormone activity at the level of metabolism, transport, perception, and signaling, the prediction of hormone regulatory network behavior and output becomes unfeasible. Modelling and mathematical simulations provide a novel means to address these issues and help to achieve better understanding of the complexity and dynamics of hormone action (Voß et al., 2014).

For example, studies of the transcription factor *PHABULOSA* (*PHB*) and cytokinin in controlling the root meristem size showed that cytokinin regulates *microRNA165/166* and that both cytokinin and *microRNA165/166* jointly regulate *PHB*. In return, *PHB* promotes cytokinin biosynthesis by stimulation *IPT7* expression (Dello Ioio et al., 2012). One-dimensional model and mathematical simulations provided insights into the functioning of such a complicated molecular network, showing that this regulatory loop restrains the reduction and accelerates the recovery of *PHB* levels thus providing robustness against cytokinin fluctuations (Dello Ioio et al., 2012).

A combination of experimental and modelling approaches has also been applied to integrate auxin and cytokinin pathways in the specification of vascular patterning. A two-dimensional multicellular model of Muraro et al., 2014 incorporated previous findings of a mutually inhibitory interaction between auxin and cytokinin, mediated through the auxin inducible repressor of the cytokinin signaling *AHP6*; cytokinin feedback on the PIN auxin efflux carriers and *SHORT ROOT* (*SHR*) promoted expression of the mobile *microRNA165/166* which silences *PHB* to form a gradient of *PHB* mRNA that controls the specification of xylem and inhibits *AHP6* expression (Bishopp et al., 2011b; Carlsbecker et al., 2010). Mathematical simulations revealed that this gene regulatory network is not sufficient to establish proper expression patterns of key marker genes as observed experimentally, and predicted additional negative regulators of cytokinin signaling and the mutual degradation of both *microRNA165/6* and *PHB* mRNA (Muraro et al., 2014).

A genetic network tested in the model simulation of De Rybel et al., 2014 integrated two incoherent feed-forward loops and evaluated their impact on the patterning of vascular tissues. One of the feed-forward loops implements auxin-cytokinin antagonistic regulations of PIN mediated auxin efflux (Bishopp et al., 2011b; Mähönen et al., 2006). A second loop is based on the experimental identification of interaction between MONOPTEROS/ARF5 and TARGET OF MONOPTEROS5 /LONESOME HIGHWAY (TMO5)/LHW) and LONELY GUY4 (LOG4) which mediates auxin-dependent control of the cytokinin biosynthesis (De Rybel et al., 2013). The authors show that the individual subnetworks provide specific regulatory inputs, one generating a high-auxin domain whereas a second defines sharp boundaries between the high auxin domain and the neighboring cytokinin response domain. Integration of both regulatory circuits is sufficient to generate distinct hormonal zones and establishment of stable patterns within a vascular tissue (De Rybel et al., 2014).

Conclusion

History of auxin and cytokinin from the initial discoveries by brothers Darwin's (1880) and Gottlieb Haberlandt (1919) is a beautiful demonstration of unceasing continuity of research. Novel findings are integrated into existing hypotheses and models and deepen our understanding of biological principles. At the same time new questions are triggered and hand to hand with this new methodologies are developed to address these new challenges.

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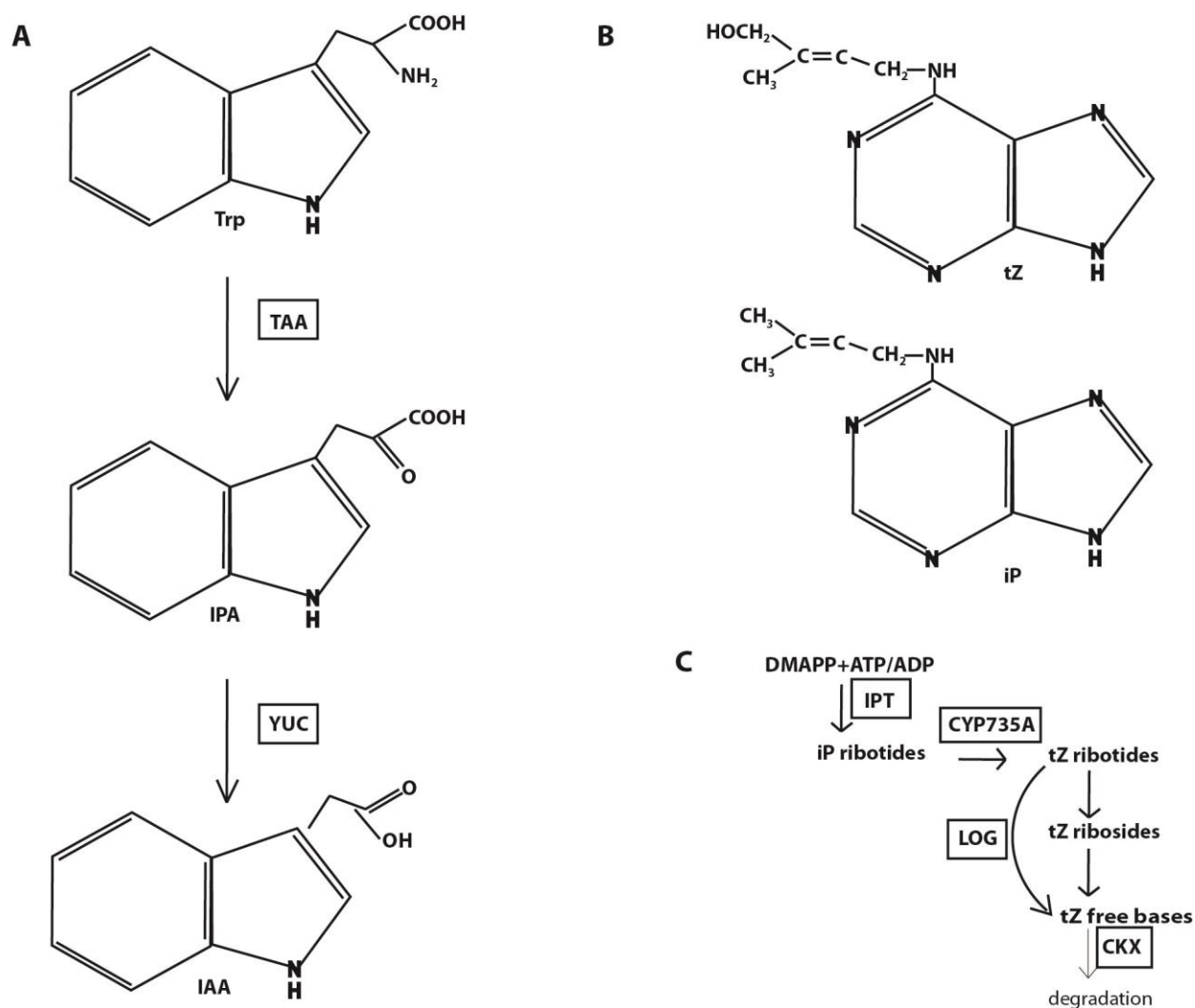


Figure 1. Biosynthesis of auxin and cytokinins. (A) Auxin (IAA) is synthesized from tryptophan (Trp) precursor in two step pathway catalyzed by TAA and YUCCA. (B) Common plant cytokinins trans-zeatin (tZ) and isopentenyl-adenine (iP). (C) Core steps of cytokinin metabolism. Biosynthesis of tZ cytokinin is initiated by adenosine phosphate-isopentenyltransferase (IPT) using dimethylallyl diphosphate (DMAPP) and adenosine 5'-diphosphate (ADP), or adenosine 5'-triphosphate (ATP) to form iP-ribotides which are converted to the corresponding tZ-ribotides by cytochrome P450 monooxygenases (CYP735As). tZ-ribotides can be dephosphorylated to tZ-ribosides or directly converted to active free bases by cytokinin nucleoside 5'-monophosphate phosphoribohydrolase (LOG).

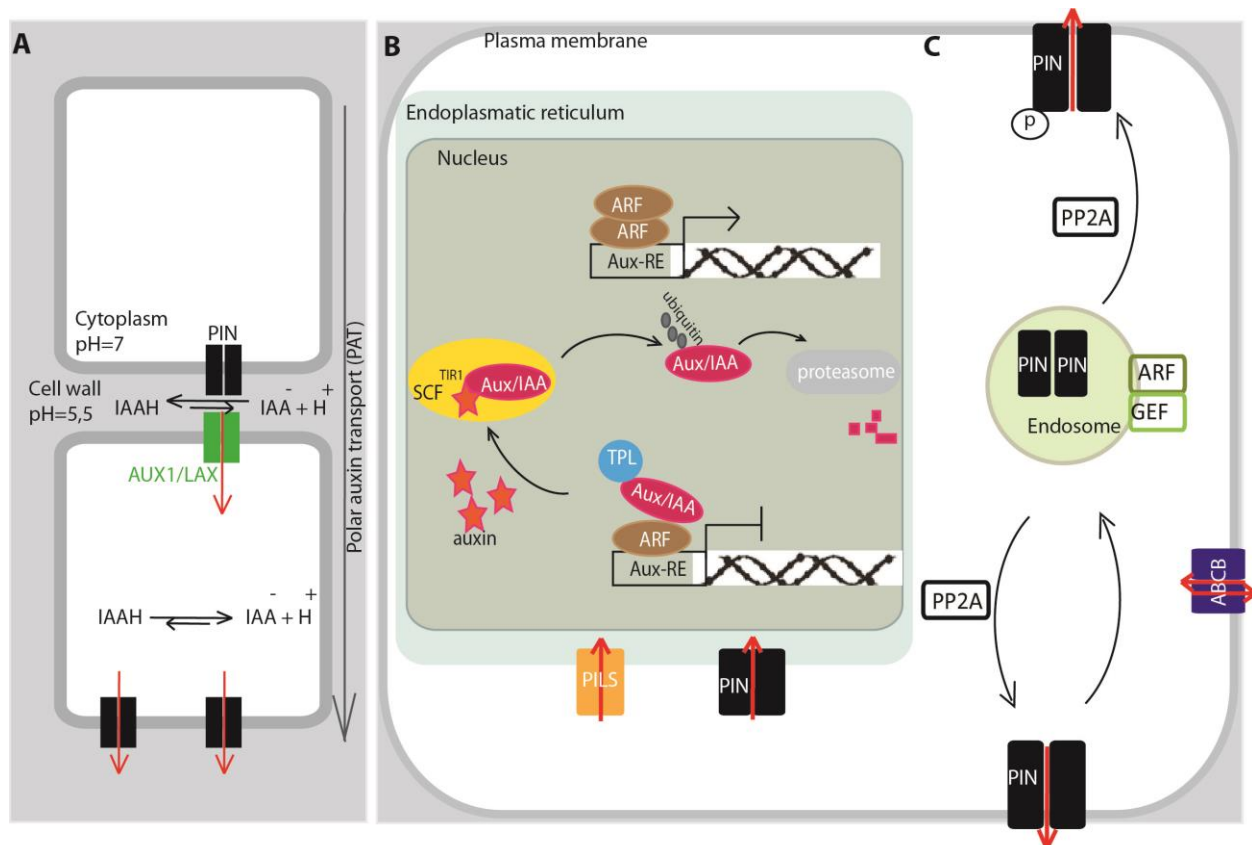


Figure 2

Figure 2. Model of auxin transport and signaling. (A) Chemiosmotic hypothesis for polar auxin transport. In the acidic apoplast auxin is protonated. The protonated auxin either passively diffuses through the plasma membrane or is actively transported by AUX1/LAX influx carriers into the cell. In the neutral cytosol auxin becomes deprotonated and can leave the cell only by auxin efflux carriers such as PIN proteins and PGP transporters. (B) Under low auxin conditions, Aux/IAAs form a complex with ARF transcription factors and the TPL corepressor, thus thereby inhibiting AuxRE-mediated gene transcription. At higher concentrations, auxin stimulates ubiquitin-mediated proteolysis of Aux/IAA catalysed by an SCF^{TIR1} E3 ubiquitin ligase. Degradation of Aux/IAAs relieves the ARF repression and allows transcription. (C) Outside the nucleus PIN auxin efflux transporters cycle between endosomes and the plasma membrane. The exocytosis requires the activity of GNOM, an ADP-ribosylation factor GTPase guanine nucleotide exchange factor (ARF-GEF), whereas endocytosis occurs in a clathrin-dependent manner. The PIN phosphorylation status, controlled by PINOID kinase (PID) and protein phosphatase 2A (PP2A), determines PINs recruitment to apical or basal targeting pathways. The short PIN proteins and PILS located in the ER might regulate of intracellular auxin homeostasis.

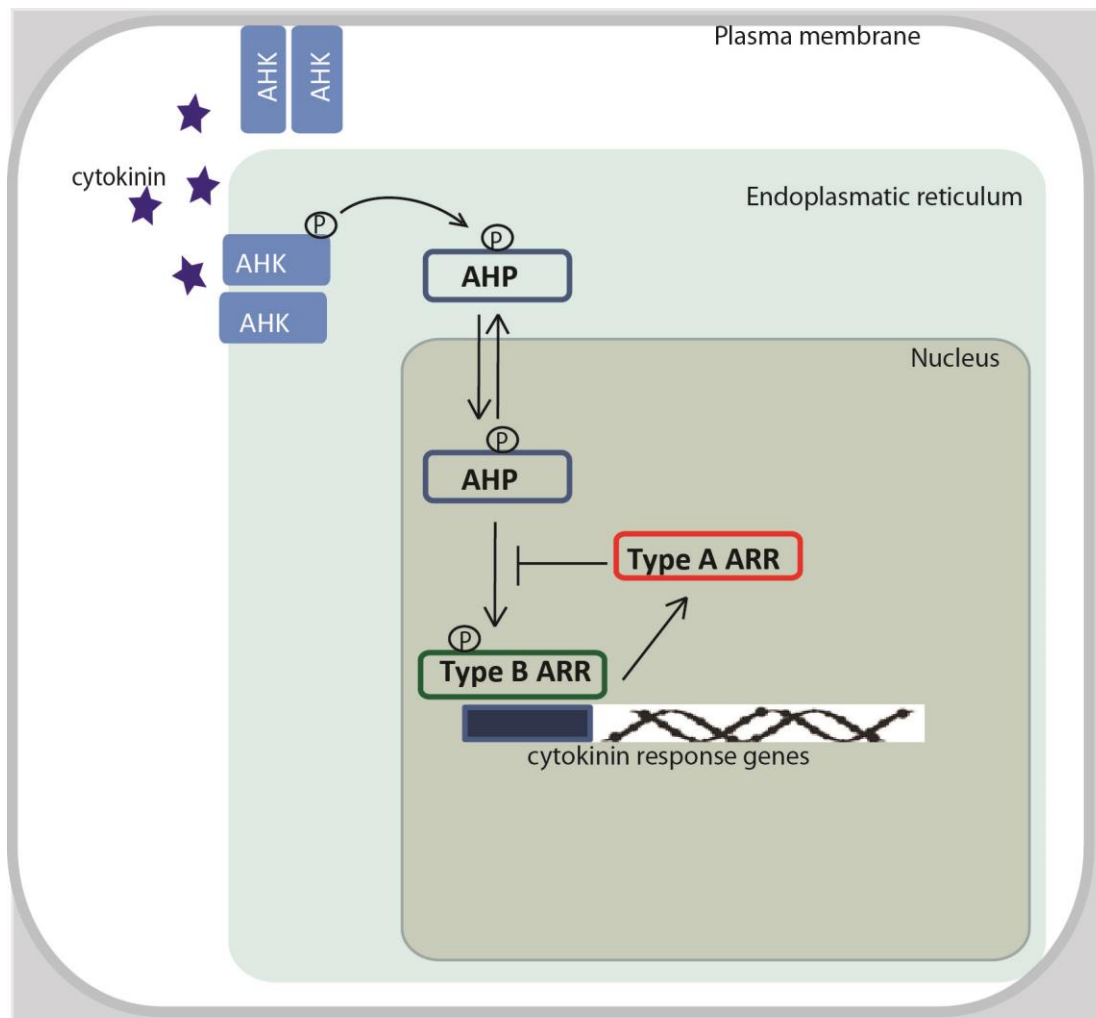


Figure 3

Figure 3. Model of cytokinin signaling pathway. Cytokinin binds to cytokinin receptor (AHKs) and initiates the phosphorelay signal transduction cascade. The phosphate is transferred from receptor to histidine phosphotransfer proteins (AHPs) followed by the phosphorylation and activation of the type B response regulator (ARR) proteins in the nucleus. A negative feed-back loop is provided by type-A ARRs, which inhibits the activity of type-B ARRs.